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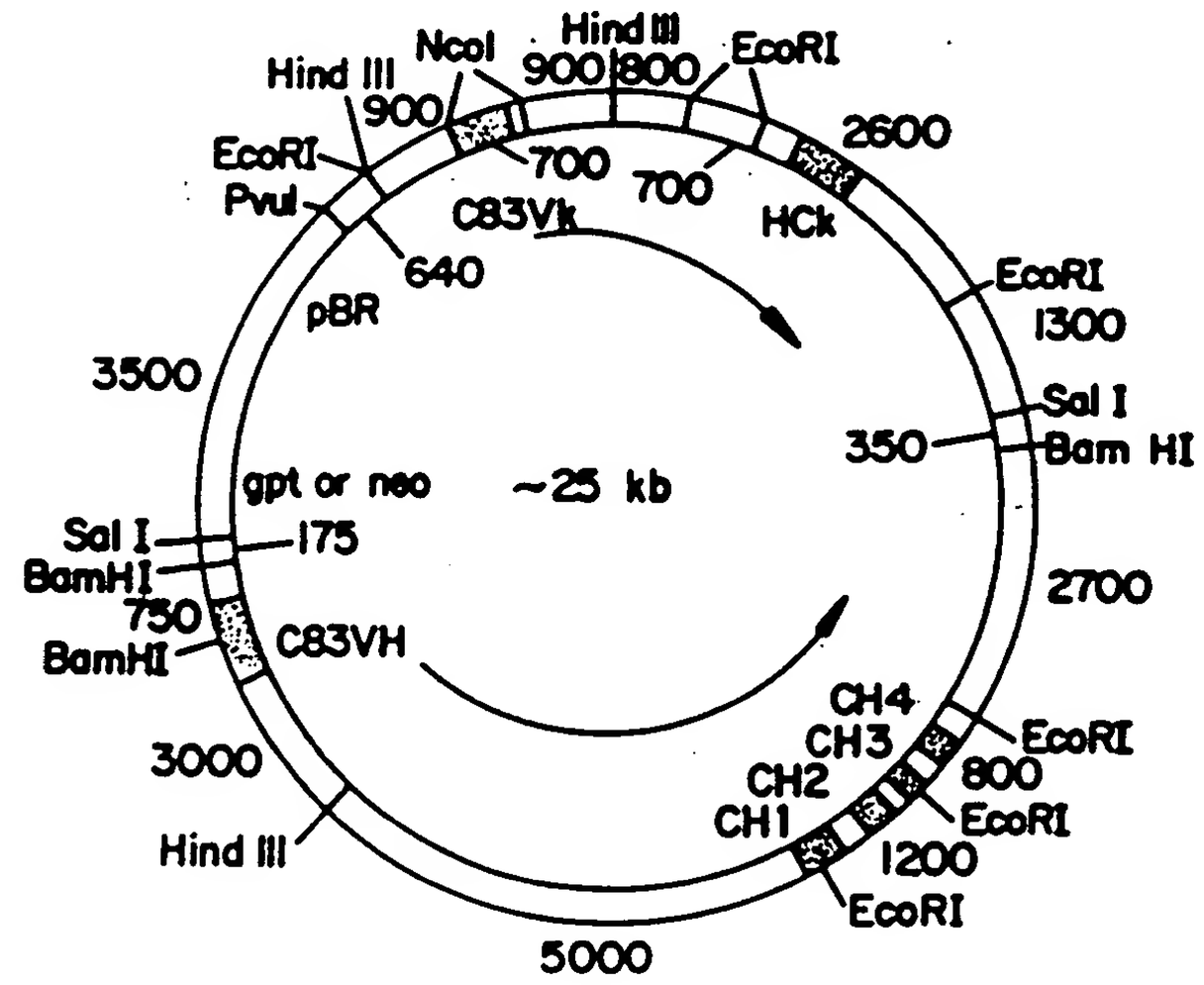

(57) Abstract

Human monoclonal antibodies are expressed by recombinant mammalian cells transformed with vectors containing genes encoding the heavy and light chains of the human monoclonal antibodies. The genes were isolated from a genomic library constructed from a human/human or human/murine hybridoma which produces the antibodies.

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Method for Improving Human Monoclonal Antibody Production

Background

The successful utilization of human monoclonal antibodies for treatment and diagnosis of human disease is potentially limited by difficulties associated with their commercial production. Although murine monoclonal antibodies are relatively easy to generate in commercial quantities, they have been associated with immunogenicity in humans which reduces their effectiveness *in vivo*. Development of an immune response against mouse proteins can neutralize the therapeutic effects of murine monoclonal antibodies and trigger potentially dangerous anaphylactic reactions.

Human monoclonal antibodies offer several advantages over murine monoclonal antibodies, particularly as potential pharmaceutical agents. For example, human monoclonal antibodies are not likely to provoke an immune response comparable to the human anti-mouse antibody (HAMA) response typically seen after administration of murine antibodies. A HAMA response may accelerate clearance of a circulating monoclonal antibody and block its binding to an antigen thereby reducing its effectiveness. Human monoclonal antibodies may be more effective in interacting with the human immune system to activate therapeutically useful effector functions, such as antibody-dependent cellular cytotoxicity, phagocytosis or complement fixation.

Human antibodies also represent a different repertoire of specificities, and therefore may recognize epitopes not detected by foreign antibodies. The effectiveness of human antibodies is less likely to be compromised by problems associated with immunogenicity such as immune complex formation or anaphylaxis.

In spite of the many advantages of human monoclonal antibodies for therapy, their potential has not been realized, primarily because technical obstacles have limited the number and quality of cell lines that secrete them. Human hybridoma technology suffers from a number of technical shortcomings. Hybrids formed by fusion of mouse myeloma cells with human lymphoid cells display a preferential loss of human chromosomes making it difficult to achieve long-term production of human monoclonal antibodies. The use of human myeloma cell lines as fusion partners has been successful in some cases but there remains a problem with long term stability. Human monoclonal antibodies are difficult to generate and produce in commercial quantities because of these problems. Antibodies that have been generated, either from totally human hybridomas or human-rodent heterohybridomas can be produced only at low levels. These hybridomas often suffer from low antibody production and instability of the antibody-producing phenotype, presumably due to loss of crucial chromosomes.

Clinical immunology research and <u>in vivo</u> therapy would benefit greatly if methods were

available for reproducibly generating human monoclonal antibodies.

Summary of the Invention

The present invention provides a method for producing selected human monoclonal antibodies using recombinant DNA technology. In this method, regions of human immunoglobulin genes, generally derived from a hybridoma, are cloned and expressed in a host cell. The method involves preparing a first nucleic acid sequence which encodes a human heavy chain of the selected monoclonal antibody and a human regulatory sequence, and a second nucleic acid sequence which encodes a human light chain of the selected monoclonal antibody and a human regulatory sequence. The first and second nucleic acid sequences are used to transform a host cell. The transformed host cells are cultured under conditions appropriate to selectively grow the transformed cells. Recombinant monoclonal antibodies produced by the transformed host cell are then recovered.

The recombinant monoclonal antibodies produced by the method are substantially identical to the original human monoclonal antibodies, i.e., the monoclonal antibodies produced by the hybridoma. Transformed host cells produced by the present method are stable over many generations, and produce higher quantities of the human monoclonal antibody than the original hybridomas.

The present method has several advantages, including improved production levels of the

monoclonal antibody and stability of the transformed host cells. The antibody genes are stably integrated into the host cell genome and can be maintained in the transformed host cell, for example, by appropriate drug selection, resulting in a high level of expression of recombinant human monoclonal antibodies. The present method allows the monoclonal antibody isotype to be selected on a rational instead of a random basis. Thus, the isotype of the human monoclonal antibody can be chosen to maximize therapeutic effectiveness.

Brief Description of the Figures

Figure 1 is a schematic representation of the DNA probes J_H and J_K used to screen the HA-1A library.

Figure 2 is a schematic representation of plasmids pSV2neoHuk(S) and pSV2gptHuk(S).

Figure 3 is a graph showing the results of a lipid A binding assay which shows that binding of the monoclonal antibody produced by the 148 cell line is indistinguishable from the binding of the original HA-1A C83 antibody.

Figures 4A and 4B are graphs showing the results of experiments comparing the C83 cell line with the 148 cell line for antibody production (4A) and cell number (4B).

Figure 5 is a schematic representation of plasmids pSV2gptC83DP and pSV2neoC83DP.

Detailed Description of the Invention

In the method of the invention, nucleic acid sequences, or genes, are prepared which encode human heavy and light chains of a selected monoclonal antibody, and human regulatory nucleotide sequences specific for the genes. Alternatively, the first and second nucleic acid sequences can encode just the variable regions of the heavy and light chains. The first nucleic acid sequence encodes all or a portion or the heavy chain and its associated regulatory sequence, and the second nucleic acid sequence encodes all or a portion of the light chain and its associated regulatory sequence. The genes are generally derived from a hybridoma which produces the selected monoclonal antibody but can be obtained from other sources which contain the nucleotide sequences encoding the antibody proteins. The genes can be derived from different hybridomas, that is, the light chain sequence from one hybridoma and the heavy chain sequence from a different hybridoma. The hybridoma can be any human/human or human/murine hybridoma of choice which produces the selected human monoclonal antibody.

As used herein, the term "human/human hybridoma" means fused hybrid cell lines created by fusing a B lymphocyte with a long-lived neoplastic plasma cell, or a T lymphocyte, with a lymphoma cell, wherein the fusion partners of both are of human origin. A "human/ murine hybridoma" refers to fused hybrid cell lines wherein one of the fusion partners is of murine origin.

The expression "nucleic acid sequence" or "nucleotide sequence" refers to a linear segment or polymer of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The polymer of DNA or RNA can be single- or double-stranded, optionally containing synthetic, non-natural, or altered nucleotides capable of incorporation into DNA or RNA polymers.

The first and second nucleotide sequences preferably include the regulatory nucleotide sequences which control the transcription and translation of the genes. The terms "human regulatory nucleotide sequence" or "human regulatory sequence", as used herein, refer to a nucleotide sequence of human origin which is located 5' and/or 3' to a nucleotide sequence the transcription of which is controlled by the human regulatory nucleotide sequence in conjunction with the protein synthetic apparatus of the cell. A human regulatory nucleotide sequence can include a promoter region, as that term is conventionally employed by those skilled in the art. A promoter region can include, for example, an association region recognized by an RNA polymerase, one or more regions which control the effectiveness of transcription initiation in response to physiological conditions, and the transcription initiation sequence and the regulatory elements required for expression of the gene (i.e., promoter, enhancer, octamer, splicing and polyadenylation signals).

In one embodiment of the present method, nucleic acid sequences encoding the intact heavy chains and light chains of the selected monoclonal antibody are isolated from a hybridoma by constructing a genomic library from the hybridoma DNA. The genomic library is constructed by well-known techniques, e.g., utilizing a bacteriophage vector, such as lambda (λ) gt10 or λ EMBL-3. The vector is then used to infect a host cell, wherein the insert DNA is amplified. In a preferred embodiment of the present method, a genomic library is prepared from the hybridoma DNA and cloned into the bacteriophage vector λ EMBL-3.

The genomic library is screened or probed to determine which segments of the DNA in the library encode the heavy and light chains of the antibody. Heavy and light chain DNA probes are used to screen the genomic library. For example, DNA probes derived from the human light and heavy chain J regions of the immunoglobulin loci (hereinafter, J_H and J_K for heavy and light chains, respectively) can be used. Other probes from the immunoglobulin loci can be used, for example, constant region sequences from the heavy and light chain genes. Screening of the DNA library can be performed as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1982).

DNA clones which have been identified as containing the desired human heavy and light chain genes are isolated from the genomic library. Restriction endonuclease maps of the clones are

constructed and compared to published maps to verify that they are derived from the immunoglobulin loci. Ravetch et al., Cell, 27:583-591 (1981); Klobeck and Zachaw, Nucleic Acids Research, 14:4591-4603 (1986). The variable regions can be isolated and used as probes in Northern blot analysis (Maniatis et al., supra.) to verify that these sequences are expressed in the original hybridoma.

The isolated nucleic acid sequences are sub-cloned by standard techniques into vectors, e.g., plasmid or viral vectors. Intact heavy and light chain genes can be cloned into separate plasmids or combined into a single large plasmid. If a different isotype is desired, or if only the variable region is cloned for the heavy or light chain, the variable region alone is cloned into vectors containing previously cloned constant regions.

The regulatory elements required for expression of the antibody genes in the host cell (i.e., promotor, enhancer, octamer, splicing and polyadenylation signals) are generally present on the cloned DNA, as part of the regulatory sequences. In addition to the nucleic acid sequences encoding the heavy and light chains and associated regulatory sequences, the vector can also include a marker gene for selection in mammalian cells, which allows the host cells expressing the foreign DNA to grow in selective media. For example, the plasmid vectors pSV2gpt (ATCC No. 37145) or pSV2neo (ATCC No. 37149) are particulary useful in the present method.

Plasmid pSV2gpt contains a gene encoding a protein necessary to confer resistance to the drug mycophenolic acid, and pSV2neo contains a gene encoding a protein necessary to confer resistance to the drug G418. To allow growth in bacterial cells for ease of manipulation, an antibiotic resistance gene such as Amp^r (ampicillin resistance) can be included in the vector. Plasmids which are particularly useful are plasmids containing the human CK regions. Oi and Morrison, Biotechniques, 4:214-221 (1986).

The vectors containing the cloned antibody genes are transfected into a suitable host cell line by any known transfection method, e.g., electroporation, calcium phosphate precipitation, DEAE-dextran, or protoplast fusion. Any mammalian cell that supports high levels of synthesis and secretion of functional antibody molecules can be used as a host cell. A well characterized, non-producing murine myeloma cell line is particularly useful as a host. A preferred host cell line is a murine myeloma cell line which lacks any non-characterized human DNA, viral nucleic acids or viral particles. Utilizing a murine myeloma host cell reduces or eliminates the possibility of contamination of the human monoclonal antibody preparation with human viruses or uncharacterized human DNA, which could occur if a human host cell was used. Examples of suitable murine myeloma host cells include SP2/O-Ag14 and P3X63-Ag8.653, both of which are available from the American Type Culture Collection.

After transfection, the cells are grown in selective media such that only cells that have acquired the transfected DNA can survive. As

previously stated, the plasmid vector contains a selectable marker gene encoding a protein which confers resistance to certain drugs on transformed cells containing the gene. Suitable selective drugs include G418, mycophenolic acid and hygromycin.

Cell clones that express or secrete antibody are identified, for example, by assaying the culture supernatant for the presence of antibody by any conventional assay technique, such as enzyme-linked immunosorbent assay (ELISA) or particle concentration fluorescence immunoassay. Monoclonal antibodies produced by the transformed cell line can be purified by conventional techniques, such as protein A affinity chromatography or standard biochemical procedures.

In one embodiment of the present invention, a library of genomic DNA fragments was constructed from the DNA of a human-derived hybridoma which produces a human monoclonal antibody specific for the lipid A portion of a bacterial gram-negative lipopolysaccharide. The genomic library was constructed in a bacteriophage vector, λ EMBL-3, using standard recombinant DNA techniques. The library was screened with a J region probe from the human kappa (κ) locus and a J region probe from the human heavy chain locus. These probes hybridized with the rearranged human light and heavy chain genes. Positive clones containing the variable regions of the heavy and light chains were isolated and characterized by restriction enzyme mapping.

The light chain variable region was cloned into a plasmid vector already containing the human κ region. The entire heavy chain gene, including variable and μ constant regions, was cloned into the resulting plasmid, so that functional antibody genes were recreated. Two such plasmids were constructed differing only in the selectable marker which was employed (i.e., gpt and neo) to ensure the presence of at least two copies of each gene (i.e., heavy and light chain genes) in the cell line.

The plasmids were transfected into a host murine cell line, SP2/0, by electroporation. The SP2/0 cell line was chosen to maximize antibody production and to minimize potential virus contamination of the product. This cell line grows well and has previously been used to express chimeric mouse-human antibodies. The cells were cultured in media containing drugs which selectively allow growth of cells expressing the drug-resistance genes, that is, cells which have incorporated and express the plasmid DNA. Drug-resistant clones were tested for the presence of secreted antibody in the cell supernatant. The clones producing the highest level of antibody were then subcloned, and after several subcloning cycles, a stable, high-level producer was selected.

The transfected cells were stable over many generations because the human immunoglobulin (Ig) genes become integrated into the host cell genome. Drug selection can be maintained, but is not always necessary, to ensure stability of the transformed

cell lines. Some subclones maintain a high level of antibody production in the absence of drug selection.

The resulting antibody was then analyzed in the a lipid A binding assay to demonstrate its equivalence with the original human monoclonal antibody derived from the hybridoma. The binding activity of the recombinant human monoclonal antibodies produced by the transformed cells was indistinguishable from the binding activity of antibodies produced by the original hybridoma.

Monoclonal antibodies produced by the present method can be used for in vitro, clinical and/or diagnostic purposes, such as immunoassays. The present monoclonal antibodies are particularly useful for in vivo therapeutic purposes due to the increased quantities of uniform antibodies which are produced by the present method. For in vivo purposes, human monoclonal antibodies are superior to mouse immunoglobulins as they generally persist in the circulation for longer periods, and do not elicit a strong antibody response in the human. The present human recombinant monoclonal antibodies can be complexed with a drug or a cytotoxin or labeled with a radionuclide for therapeutic applications or a radionuclide for in vivo imaging of tumors, for example. Other applications include use in vaccines, or active immunization, e.g., with antiidiotypic antibodies to raise antibodies against pathogens not suitable for conventional vaccines and

modulation of autoimmune and/or endocrine conditions with antireceptor antibodies.

The present invention is further illustrated by the following Exemplification.

EXEMPLIFICATION

Materials and Methods

Cell Lines

HA-1A (cell line C83:MCB; M83-19, Centocor, Inc., Malvern, PA) is a mouse-human hybridoma generated by fusion of an EBV-transformed human splenic B-lymphocyte with a mouse-human hetero-myeloma. HA-1A secretes a human IgM,K antibody (referred to herein as "HA-1A antibody" or "HA-1A C83") with immunoreactivity towards the lipid A portion of bacterial gram-negative lipopoly-saccharide. Growth medium for HA-1A was Iscove's medium supplemented with 5% fetal bovine serum (FBS). SP2/0 cells were obtained from the American Type Culture Collection (ATCC #1581) and grown in Iscoves medium supplemented with 10% FBS.

Hybridization Probes

The DNA probes for screening the HA-1A library are shown schematically in Figure 1. The human heavy chain J_H probe is a 6.0 kb BamHI-HindIII fragment derived from the heavy chain locus containing all four J_H exons. The human light chain probe is a 1.8 kb SacI fragment from the kappa locus

containing the five J_K exons. ^{32}P-labeled probes were prepared by nick translation using a kit obtained from Amersham, Inc. (Arlington Heights, IL). Unincorporated nucleotides were removed by centrifugation through a Sephadex G-50 column. The specific activities of the probes were 5-10 x 10^8 cpm/μg.

Construction and Screening of a Genomic Expression Library

A genomic DNA library was constructed from HA-1A DNA from the cell line C83:MCB; M83-19 (hereinafter "C83"), a mouse/human heterohybridoma which produces a human anti-lipid A IgM monoclonal antibody. HA-1A genomic DNA was partially digested with restriction endonuclease Sau 3A and the resulting fragments were size-fractionated on a 10-40% sucrose density gradient. DNA fragments of approximately 15-23 kilobases (kb) were ligated to bacteriophage λ EMBL-3 arms previously digested with BamHI, and the resulting DNA was packaged *in vitro* into phage particles using Packagene (Promega Biotech, Inc., Madison, WI) according to the manufacturer's instructions. The phage packaged DNA was plated on 150 mm agar plates at a density of 30,000 plaques per plate.

The library was screened with the heavy chain and light chain J region ^{32}P-labeled probes according to the method described by Maniatis et al., *supra*. Plaque hybridizations were carried out in 5X SSC, 50% formamide, 2X Denhardt's reagent, 200

μg/ml denatured salmon sperm DNA at 42°C for 18-20 hours. Final washes were in 0.5X SSC, 0.1% SDS at 65°C. Positive clones were identified after autoradiography and isolated after at least three rounds of plaque purification.

DNA Sequencing

The clones were characterized by restriction mapping using standard methods as described by Maniatis et al., supra. Restriction endonuclease maps were determined for HA-1A V_H and V_K clones. Appropriate DNA fragments were subcloned into MP18 and MP19 and sequenced using the dideoxy method, with Sequenase (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. The amino acid translations were compared with previously sequenced human light and heavy chains. A Northern blot analysis showed that the cloned V regions hybridized with the appropriate size RNAs from the original C83 cell line, demonstrating that the cloned sequences were expressed in C83.

DNA Transfection using Electroporation

The light chain V region was then cloned into a plasmid expression vectors containing the human CK region and selectable markers, gpt, which confers resistance to the drug mycophenolic acid, and neo, which confers resistance to the drug G418. The starting plasmids, pSV2gptHCK (s) and pSV2neoHCK (s), are shown schematically in Figure 2. These plasmids were prepared from plasmids pSV2gpt (ATCC No. 37145) and pSV2neo (ATCC No. 37149) and a human CK region. The human CK regions were obtained from Sherie Morrison and are described by Oi and Morrison in Biotechniques, 4:214-221 (1986).

A second clone was isolated that contained an entire rearranged human heavy chain gene of the IgM type. Restriction enzyme mapping and DNA sequencing confirmed its similarity to other human antibody sequences. In addition, the variable region (V) sequence was shown to be expressed in the C83 cell line by Northern blot analysis. This gene was subcloned into the expression vectors already containing the light chain gene segments, resulting in two plasmids, pSV2gptC83DP and pSV2neoC83DP, with the potential for expressing both heavy and light chain genes derived from C83. A schematic representation of the plasmids is shown in Figure 5.

Plasmid DNA to be transfected was purified by centrifugation to equilibrium in ethidium bromide/cesium chloride gradients two times. 10-50 μg of plasmid DNA was added to 10^7 SP2/0 cells in phosphate buffered saline (PBS) on ice, and the mixture was placed in a Biorad electroporation apparatus. (BioRad Laboratories, Richmond, CA) Electroporation was performed at 200 volts and the cells were plated out in 96 well microtiter plates. Drug selection was applied after 48 hours and drug resistant colonies were identified after 1-2 weeks. Selective medium included 0.25 μg/ml mycophenolic acid, 1.25 μg/ml hypoxanthine, 25 μg/ml xanthine, and 0.5 mg/ml G418.

Quantitation of Antibody Production

Tissue culture supernatant was analyzed for human IgM protein content by ELISA assay using goat

anti-human IgM Fc5u antibody (Jackson Laboratories, Avondale, PA). Human IgM (Jackson Laboratories) was used to generate a standard curve.

Partial Purification of Recombinant HA-1A Antibodies

The recombinant antibody was purified by addition of 4% (w/v) polyethylene glycol (MW 6000) to clarified cell supernatant. After gentle mixing overnight at 4°C, the precipitate was collected by low speed centrifugation and solubilized in 0.05 M Tris/0.2 M NaCl/0.1M glycine, pH 8.6. The sample was then dialyzed against 0.3 M NaCl, 0.01 M sodium phosphate, pH 7.2.

Lipid A Binding Assay

Lipid A binding activity was determined using a solid phase ELISA assay with monophosphoryl lipid A from S. minnesota R595 (RIBI Immunochem Research, Inc., Hamilton, MT) coated on microtiter plates and an alkaline phosphatase-conjugated goat anti-human IgM antibody (Jackson Laboratories).

RESULTS

Cloning of the HA-1A Heavy and Light Chain Genes

Several positive clones were isolated from an HA-1A genomic λ EMBL-3 library using either heavy or light chain probes. Following at least three rounds of plaque purification, bacteriophage DNA was isolated, digested with various restriction enzymes, and fractionated on 1% agarose gels to generate

physical maps of the clones. The DNA was transferred to nitrocellulose and the blots were hybridized with J_H or J_K ^{32}P-labeled probes to determine which fragments contained the adjacent variable regions. For the light chain, a 2.5 kb HindIII fragment was identified that hybridized to the J_K probe. This fragment was used as a ^{32}P-labeled probe to verify that the V region sequence was expressed as RNA in the HA-1A hybridoma by Northern analysis. The 2.5 kb HindIII fragment was subsequently used for expression of the HA-1A light chain variable region. Restriction enzyme mapping indicated a heavy chain clone contained an entire rearranged μ heavy chain gene. This region was excised from the λ EMBL-3 phage using flanking SalI sites in the vector, and the 14 kb SalI fragment was used to express the HA-1A heavy chain.

Sequence Analysis of HA-1A Variable Regions

The nucleotide sequences of the HA-1A antibody heavy and light chain variable regions were determined along with the deduced amino acid sequences, and were compared to the nucleotide sequences of the heavy and light chain variable regions of the recombinant 148 antibody. Comparison of the deduced HA-1A light chain sequence with human and mouse variable region sequence indicated that the light chain of HA-1A is a member of the human V_k IIIb subgroup, and is the result of joining of the V regions to the J_{K5} exon. The HA-1A heavy chain sequence reveals that it is a member of the human

V_HII subgroup, and utilizes the human J6 exon. The deduced amino acid sequences of the 148 antibody are clearly human in character, and the first 95 amino acids of the light chain are identical to the sequence of a human IgM antibody, and the first 20 residues of the N-terminal amino acid sequences HA-1A antibody are identical to the seqeunce encoded in the recombinant light chain DNA.

Construction of Expression Plasmids

The 2.5 kb HindIII fragment containing the putative HA-1A light chain variable region was cloned into the HindIII sites of plasmids pSV2gptHuk(S) and pSV2neoHuk(S), respectively. These plasmids contain the human CK region and dominant selectable markers gpt and neo, for selection in mammalian cells (Figure 2). The 14 kb SalI fragment containing the complete putative HA-1A heavy chain gene was cloned into the Sal I sites of the vectors containing the light chain variable region. The resulting plasmids, pSV2gpt283DP and pSV2neoC83DP, (shown schematically in Figure 5), differ only in the selectable marker employed, and contain single copies of both heavy and light chain genes. The expression plasmids were designed to direct expression of the antibody genes using the natural cis-acting regulatory sequences linked to the genes including the octamer sequences, promoters, enhancers, splice signals, and poly A addition signals.

To express the recombinant antibody, the two plasmids were cotransfected into the nonproducing murine myeloma-derived cell line SP2/0 and a double selection with mycophenolic acid and G418 was used to obtain stable transfectants. Two copies of each gene were transfected to augment the copy number in recipient cells, and to increase the likelihood of favorable integration sites. Resistant colonies were expanded and subclones were generated from the clones secreting the highest level of human IgM antibody as measured by ELISA assay. One subclone, designated cell line C83-148-3F3-14-21-31 (hereinafter, the "148" cell line or "C83/148"), was chosen for further study.

Characterization of 148 Recombinant Antibody

HA-1A antibody is a pentameric IgM molecule. Proper assembly and secretion of pentameric IgM encoded by transfected genes requires elements not supplied by the heavy and light chain gene constructs themselves. To obtain pentameric recombinant IgM, such elements must be present in the recipient cell line. To determine whether the recombinant 148 antibody synthesized in SP2/0 cells is pentameric, purified 148 antibody was passed over an HPLC gel filtration column (Dupont Zorbax GF 450), and the elution profile compared to that of a standard human IgM pentameric antibody. The major 148 peak eluted at the same position as the standard (HA-1A) IgM indicating that the recombinant antibody is of a similar size to the standard.

HA-1A antibody binds to the lipid A portion of the lipopolysaccharide (LPS) molecule derived from gram negative bacteria. To ascertain whether the 148 recombinant antibody retains the binding characteristics of HA-1A antibody, an immunoassay was performed using purified HA-1A from the original hybridoma, C83 (the C83 antibodies) and the 148 antibodies. Figure 3 shows the results of the lipid A binding assay. The binding curves for the two antibodies are indistinguishable, demonstrating that the 148 antibody (white circles) is equivalent to the C83 antibody (white squares) in the lipid A binding assay.

Characteristics of Cell Line 148

Figure 4 shows the results of an experiment in which the C83 cell line was compared to the 148 clone with regard to antibody production and cell number. The experiment was performed under growth conditions previously determined to be optimal for C83 (i.e., Iscoves's medium supplemented with 10% FBS). The amount of antibody produced by 148 and C83 are comparable (Figure 4A) even though 148 cells grow to a lower density under conditions optimized for C83 growth (Figure 4B). These results show that the 148 antibody production per cell is significantly higher than that of C83. At the 5 day point, when cell number is maximum for both cell lines, clone 148 produces 23 $\mu g/ml/10^6$ cells whereas C83 produces 6.8 $\mu g/ml/10^6$ cells. As higher 148 cell densities are achieved, production of the

recombinant antibody would also be expected to increase.

To assess the stability of the antibody-producing phenotype for clone 148, the cells were cultured in the absence of the selective agents used during the initial selection after transfection. Antibody production decreased approximately 50% over the course of 60 days in the absence of selection. Subclones of clone 148 were obtained from the cultures that had been grown for 20 passages (approximately 60 days) without selection. A subclone, 148-35, exhibited growth and antibody production characteristics similar to 148 but is routinely cultured in the absence of selective agents. These data indicate that cell lines expressing transfected antibody genes can be obtained that do not require continued selective pressure to maintain high levels of antibody production.

DISCUSSION

The present method demonstrates the feasibility of stabilizing and improving expression of human monoclonal antibodies by cloning the antibody genes, e.g., from a hybridoma, and expressing them in a new environment suitable for long term expression. In the embodiment illustrated in the Exemplification, a mouse-human heterohybridoma, HA-1A (C83), was used as the source of human antibody genes. The heavy and light chain antibody genes were cloned using human J region probes to screen genomic libraries

made from HA-1A. The HA-1A light chain variable region was assembled with a previously cloned human kappa constant region and the entire HA-1A heavy chain gene into expression vectors containing selectable marker genes for mammalian cells. Although in this case the entire heavy chain gene was cloned, it is possible to use just the heavy chain variable region to assemble with a previously cloned constant region of the desired isotype to yield an antibody with the desired characteristics. Appropriate selection of the isotype can be useful to maximize the therapeutic effectiveness of the antibody. For example, the human IgG_1 isotype has been shown to be the most effective human isotype in mediating ADCC killing of human tumor cells. A cancer therapy monoclonal antibody, therefore, might be more effective when in the form of an IgG_1.

The transcriptional units in the expression plasmids utilized the natural human regulatory signals such as promotors, enhancers, and poly-adenylation sequences. After transfection of the expression plasmids into the mouse myeloma cell line SP2/0, a cell line was isolated, designated 148, that secreted human IgM as determined by ELISA assay.

The antibody secreted by clone 148 appears to be equivalent to the HA-1A antibody secreted by the C83 hybridoma. The deduced amino acid sequences from the cloned DNA are clearly human in character when compared to other mouse and human antibody sequences. The first 95 amino acids of the light

chain are identical to the sequence of a human IgM,K rheumatoid factor, although a different J exon is utilized. F. Goni et al., J. Immunol., 135:4073-4079 (1983). Light chains of this subgroup (V_KIIIb) comprise approximately 15% of normal Ig. D.K. Ledford et al., J. Immunol., 131:1322-1325 (1983). A partial N-terminal amino acid sequence has been performed on the HA-1A antibody and the first 20 residues are identical to the sequence encoded in the cloned light chain DNA.

Purified recombinant 148 antibody reacted with an anti-human IgM antibody in an ELISA assay, and was indistinguishable from the original HA-1A antibody in immunoreactivity towards lipid A. The recombinant antibody appears to be the correct size for pentameric IgM as determined by HPLC gel filtration chromatography.

The successful synthesis of a human antibody in a murine cell line demonstrates that there is no inherent species-specific barrier to efficient expression of human immunoglobulin promotors and enhancers can function in murine cells. Conversely, murine SP2/0 cells contain all the necessary factors in addition to synthetic and secretory apparatus to support the expression of exogenously supplied human antibody genes. Although this cross-species compatability might have been predicted, a high degree of sequence conservation between the immunoglobulin control regions of different species does not guarantee functional equivalence. For example, the rabbit kappa enhancer region is

non-functional in mouse myeloma cells despite extensive sequence homology.

The 148 cell line that secretes the recombinant antibody has characteristics desirable for large scale antibody production. Overall IgM secretion levels are comparable to the original hybridoma and in fact exceed those of the hybridoma on a per cell basis. Although 148 antibody secretion gradually decreases in the absence of selective pressure, a subclone of 148 was obtained which produces high levels of antibody in the absence of selection for at least 60 days suggesting that large scale long term production can be achieved without the addition of expensive selective agents. The 148 cell line has also been adapted to growth in 10-fold lower fetal bovine serum than the original hybridoma while still maintaining high antibody secretion. The properties of this cell line demonstrate that the problems of low secretion and loss of production in the absence of selection often seen with expression of transfected antibody genes can be overcome.

The characteristics of the 148 cell line show that human antibody genes can be rescued from "problem" hybridomas and expressed in a format suitable for high level long-term economical antibody production. Cloning of the genes may be successful even in cases where they are present in amounts fewer than one copy per cell, e.g., if human chromosomes are being lost soon after fusion. To rescue these genes, either more plaques could be screened, or the human variable region sequences

could be amplified via polymerase chain reaction prior to cloning. The recipient cell line can be chosen for its desirable characteristics, for example favorable growth kinetics, type of growth medium, and lack of endogenous pathogens or other undesirable contaminants. While avoiding the problems of low production and instability commonly associated with human-derived hybridomas, this approach also offers the advantage of a rational choice of monoclonal antibody isotype to maximize the effectiveness of a therapeutic antibody.

Equivalents

Those skilled in the art will recognize, or be able to ascertain by no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for producing a selected human monoclonal antibody, comprising the steps of:
(a) preparing a first nucleic acid sequence encoding a all or a portion of a human heavy chain of a human monoclonal antibody and a human regulatory sequence, and a second nucleic acid sequence encoding a all or a portion of a human light chain of the selected human monoclonal antibody and a human regulatory sequence;
(b) transforming a mammalian host cell with said first and second nucleic acid sequences;
(c) culturing said transformed host cell; and
(d) recovering monoclonal antibody produced by said cultured host cell.

2. A method of Claim 1 wherein the first and second nucleic acid sequences are both derived from a hybridoma which produces the selected human monoclonal antibody.

3. A method of Claim 2 wherein the first and second nucleic acid sequences are derived from a human/human or human/murine hybridoma.

4. A method of Claim 2 wherein the hybridoma is a mouse/human hybridoma HA-1A.

5. A method of Claim 1 wherein the first and second nucleic acid sequences are derived from different human-antibody-producing hybridomas.

6. A method of Claim 1 wherein the first nucleic acid sequence encodes a variable region of the heavy chain of the monoclonal antibody, and the second nucleic acid sequence encodes a variable region of the light chain of the monoclonal antibody.

7. A method of Claim 1 wherein the first nucleic acid sequence encodes an intact region of the heavy chain of the monoclonal antibody, and the second nucleic acid sequence encodes an intact light chain of the monoclonal antibody.

8. A method of Claim 1 wherein the mammalian host cell comprises a murine myeloma cell.

9. A method of Claim 8 wherein the murine myeloma cell comprises SP2/0-Ag14 or P3X63-Ag8,653.

10. A method of Claim 1 wherein the human monoclonal antibody comprises an anti-lipid A immunoglobulin M antibody.

11. A method for producing recombinant human monoclonal antibodies comprising the steps of:
(a) preparing a first nucleic acid sequence encoding a variable region of a human

heavy chain of the antibody and a human regulatory sequence, and a second nucleic acid sequence encoding a variable region of a human light chain of the antibody and a human regulatory sequence;

(b) inserting the first and second nucleotide sequences into at least one expression vector;

(c) transfecting a mammalian host cell with the expression vector thereby transforming the host cells;

(d) selectively growing the transformed host cells; and

(e) recovering human monoclonal antibodies produced by the transformed host cells.

12. A method of Claim 11 wherein the first and second nucleic acid sequences are derived from a hybridoma which produces the human monoclonal antibody.

13. A method of Claim 12 wherein the hybridoma is a human/human or a human/murine hybridoma.

14. A method of Claim 13 wherein the hybridoma is a murine/human hybridoma comprising HA-1A.

15. A method of Claim 11 wherein the first nucleotide sequence encodes an intact human heavy chain of the antibody.

16. A method of Claim 11 wherein the second nucleotide sequence encodes an intact human light chain of the antibody.

17. A method of Claim 11 wherein the expression vector is a plasmid.

18. A method of Claim 17 wherein the plasmid vector contains a nucleotide sequence encoding a human constant region of a human light chain of the antibody.

19. A method of Claim 17 wherein the plasmid vector contains a nucleotide sequence encoding a constant region of a human heavy chain of the antibody.

20. A method of Claim 17 wherein the plasmid vector contains a selectable marker gene.

21. A method of Claim 17 wherein the selectable marker gene comprises gpt or neo.

22. A method of Claim 17 wherein the plasmid comprises pSV2gptC83DP.

23. A method of Claim 17 wherein the plasmid comprises pSV2neoC83DP.

24. A method of Claim 11 wherein the host cell comprises a murine myeloma cell.

25. A method of Claim 24 wherein the murine myeloma cell comprises SP2/0-Ag14 or P3X63-Ag8, 653.

26. A method of Claim 11 wherein the human monoclonal antibody comprises an anti-lipid A immunoglobulin M antibody.

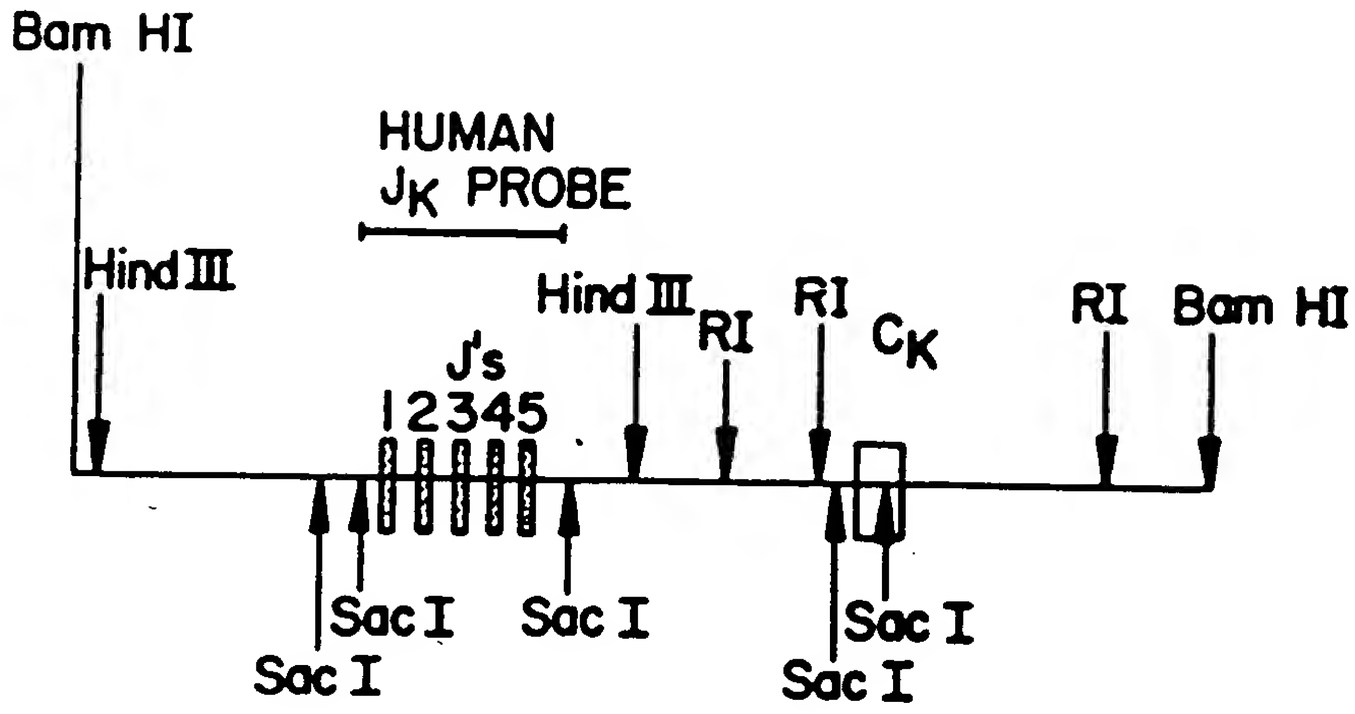
27. A murine myeloma cell transformed with the plasmid pSV2gptC83DP.

28. A murine myeloma cell transformed with the plasmid pSV2neoC83DP.

29. Cell line C83-148-3F3-14-21-31.

30. A recombinant human monoclonal antibody produced by the cell line of Claim 29.

31. A murine myeloma cell which expresses a human monoclonal antibody specific for lipid A of a gram negative bacterial endotoxin.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HUMAN
JK PROBE
Hind III
Hind III
RI
RI
CK
RI
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J's
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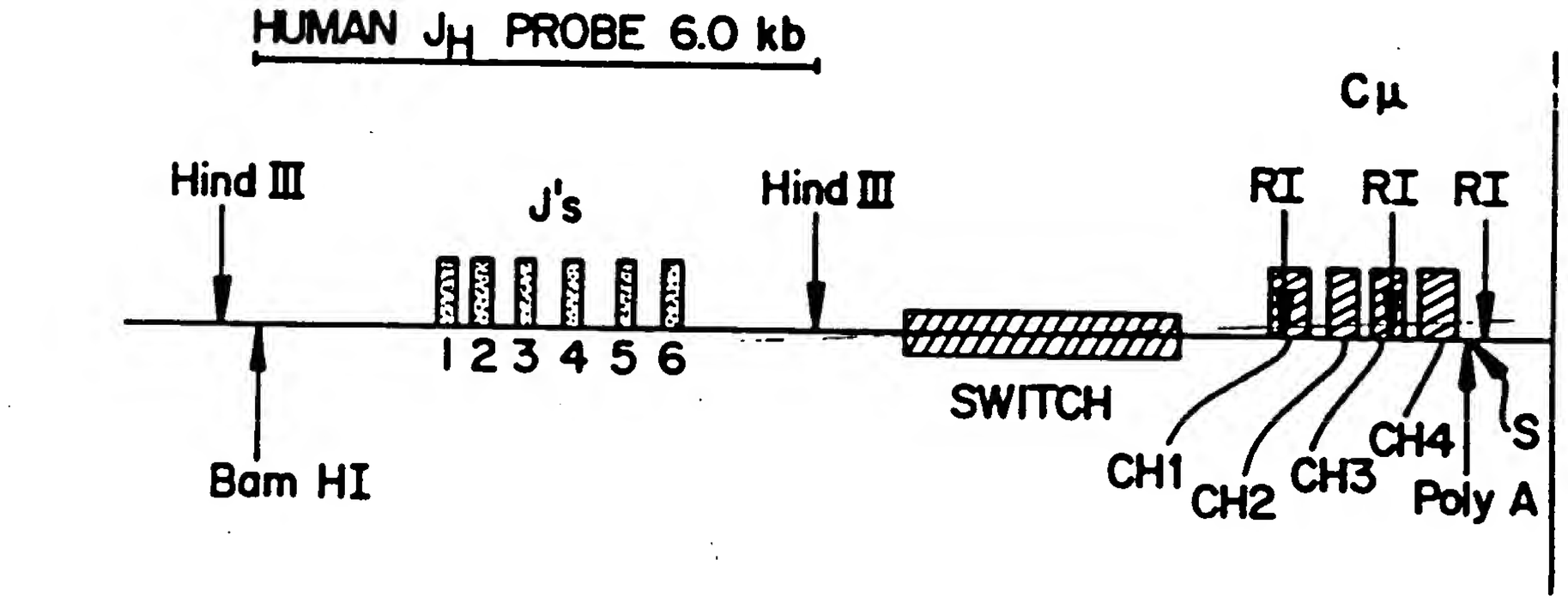
Sac I
Sac I
Sac I
Sac I
Sac I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Cμ
Hind III
Hind III
J's
RI
RI
RI
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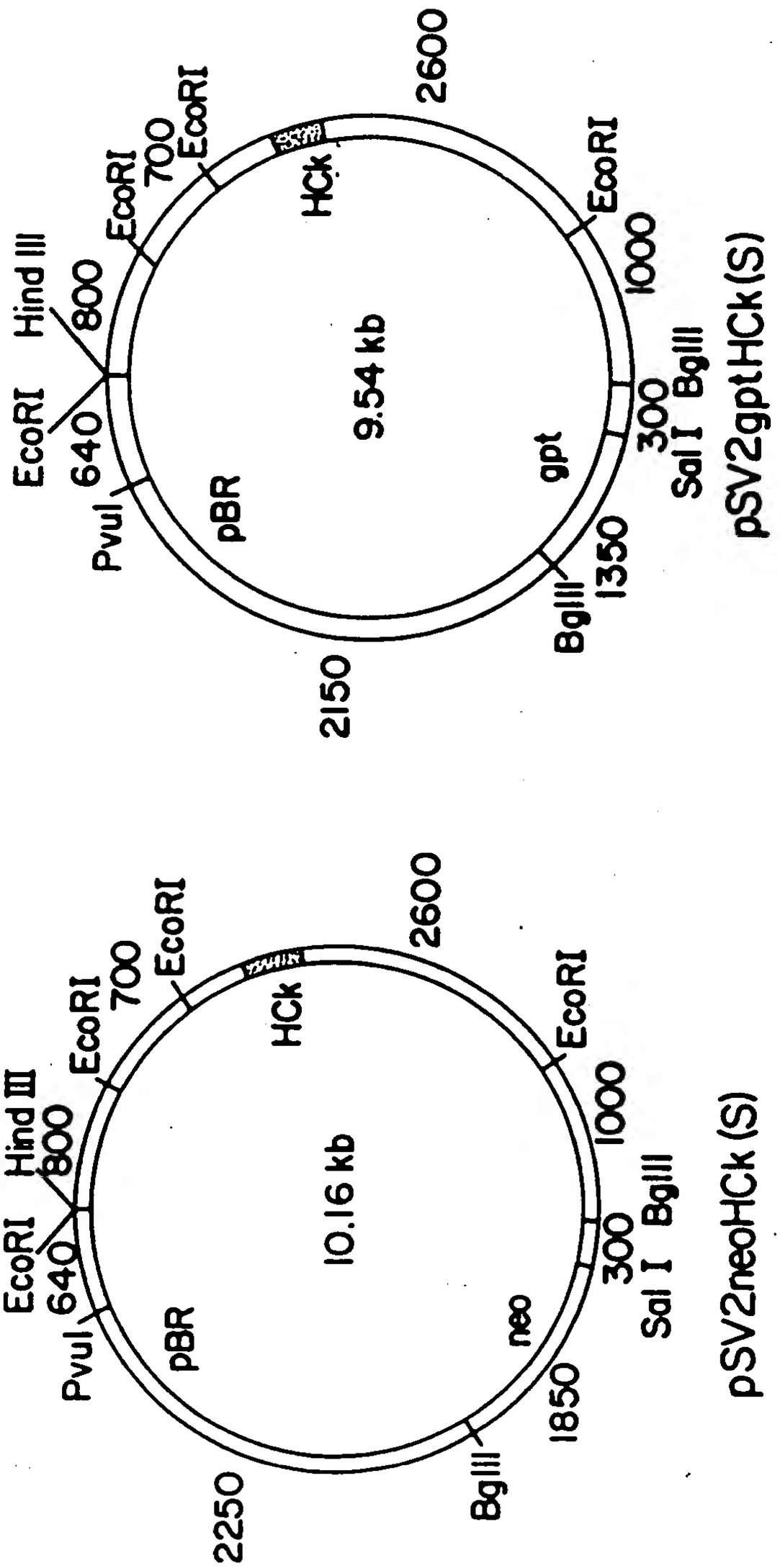
SWITCH
Bam HI
CH1
CH2
CH3
CH4
S
Poly A

FIG. 1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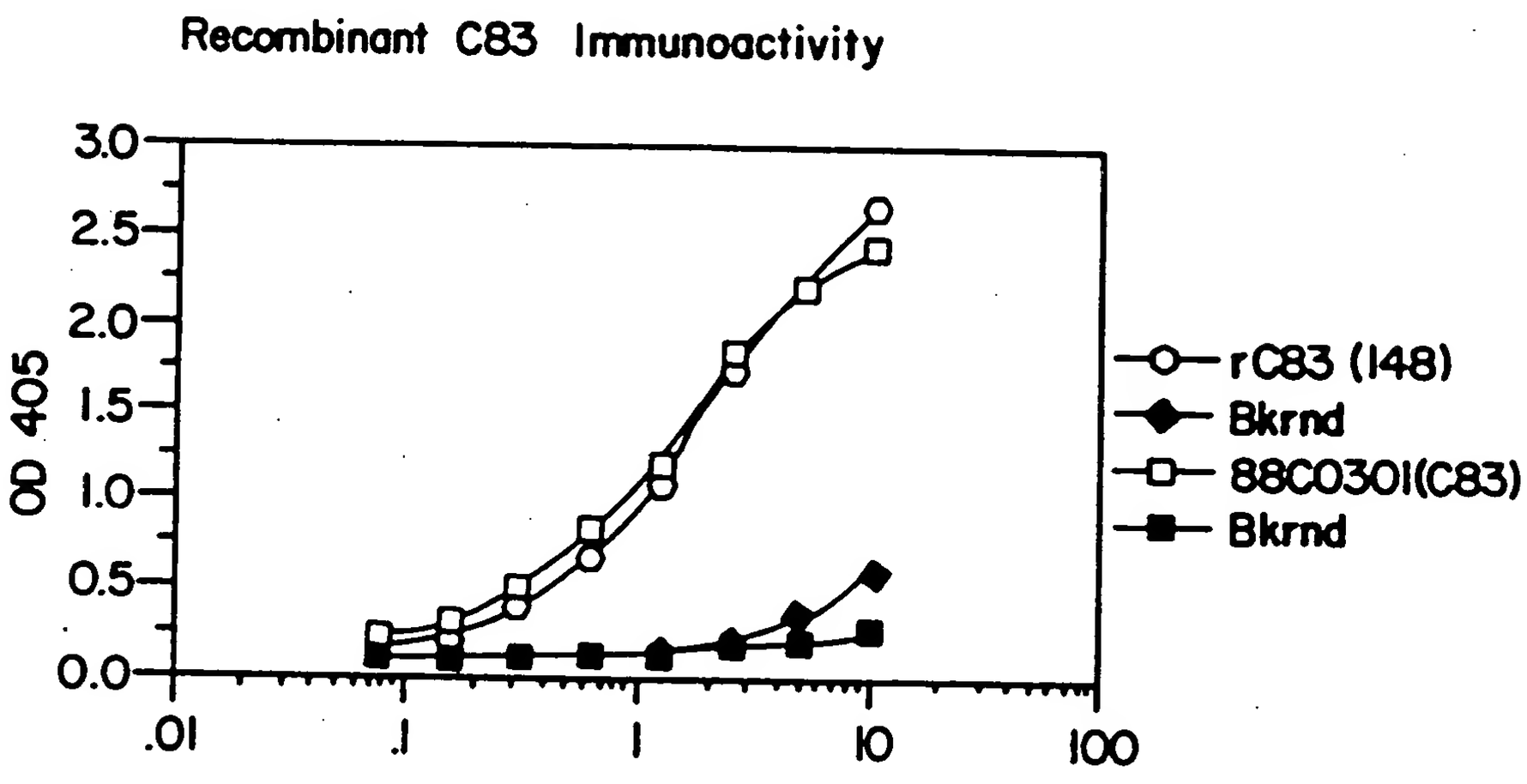
2250

FIG. 2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2.5
2.0
1.5
1.0
0.5
0.0
OD 405
.01
.1
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10
100
Conc (ug/ml)
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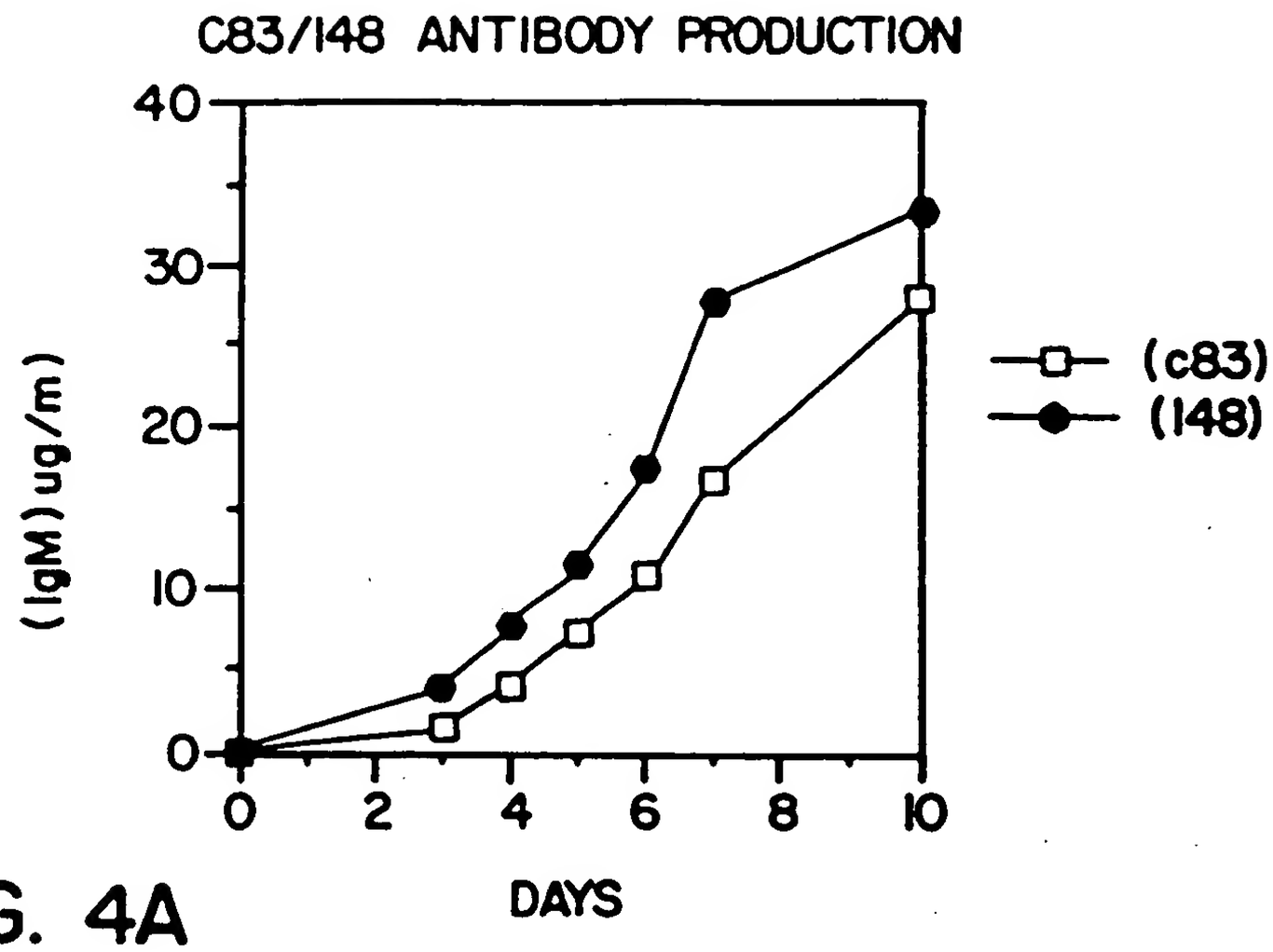
Bkrnd
88C0301(C83)
Bkrnd

FIG. 3


C83/148 ANTIBODY PRODUCTION
(IgM) ug/m
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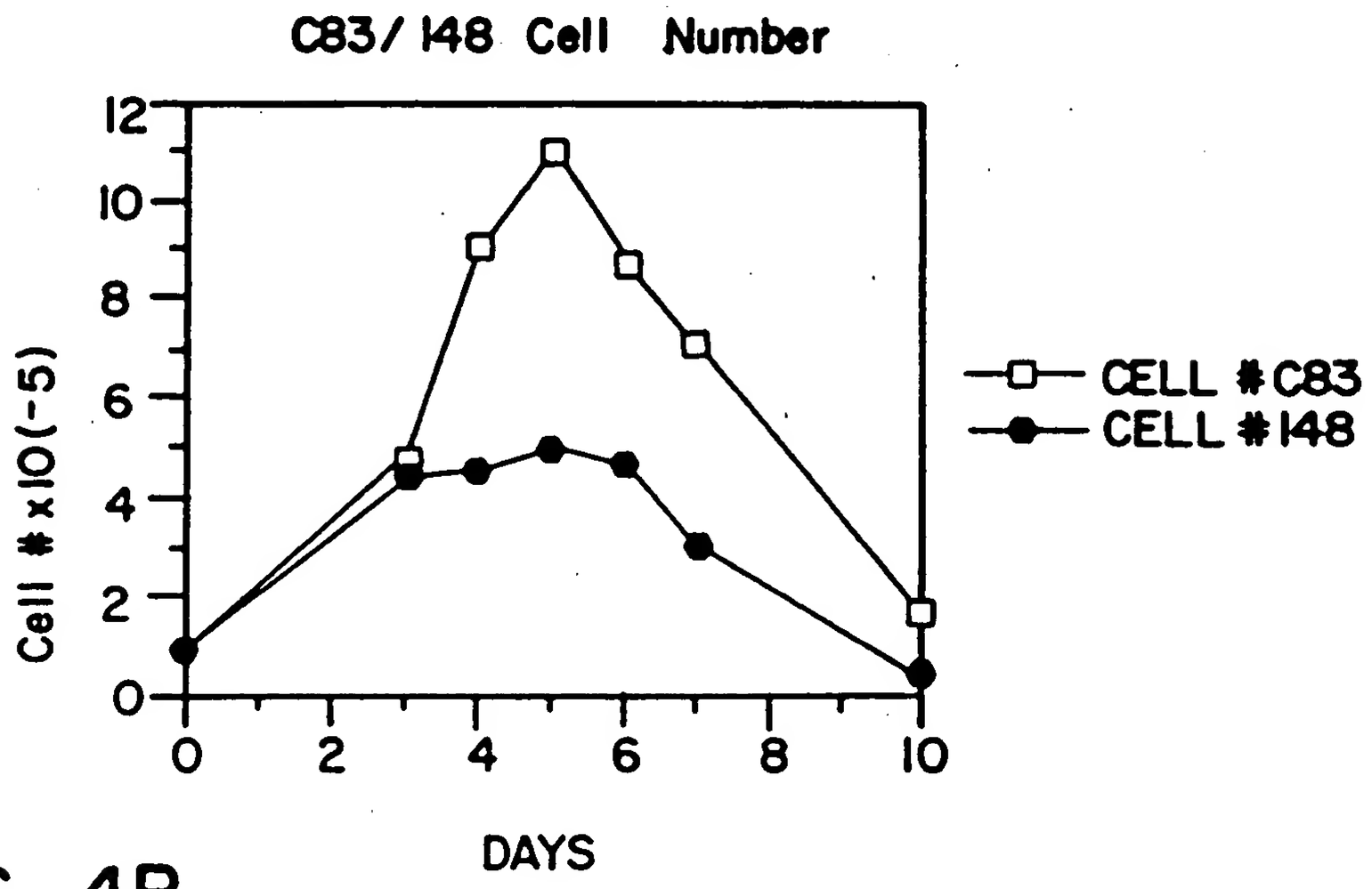
20
10
0
0
2
4
6
8
10
DAYS
(c83)
(148)

FIG. 4A


C83/148 Cell Number
Cell # x10(-5)
12
10
8
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4
2
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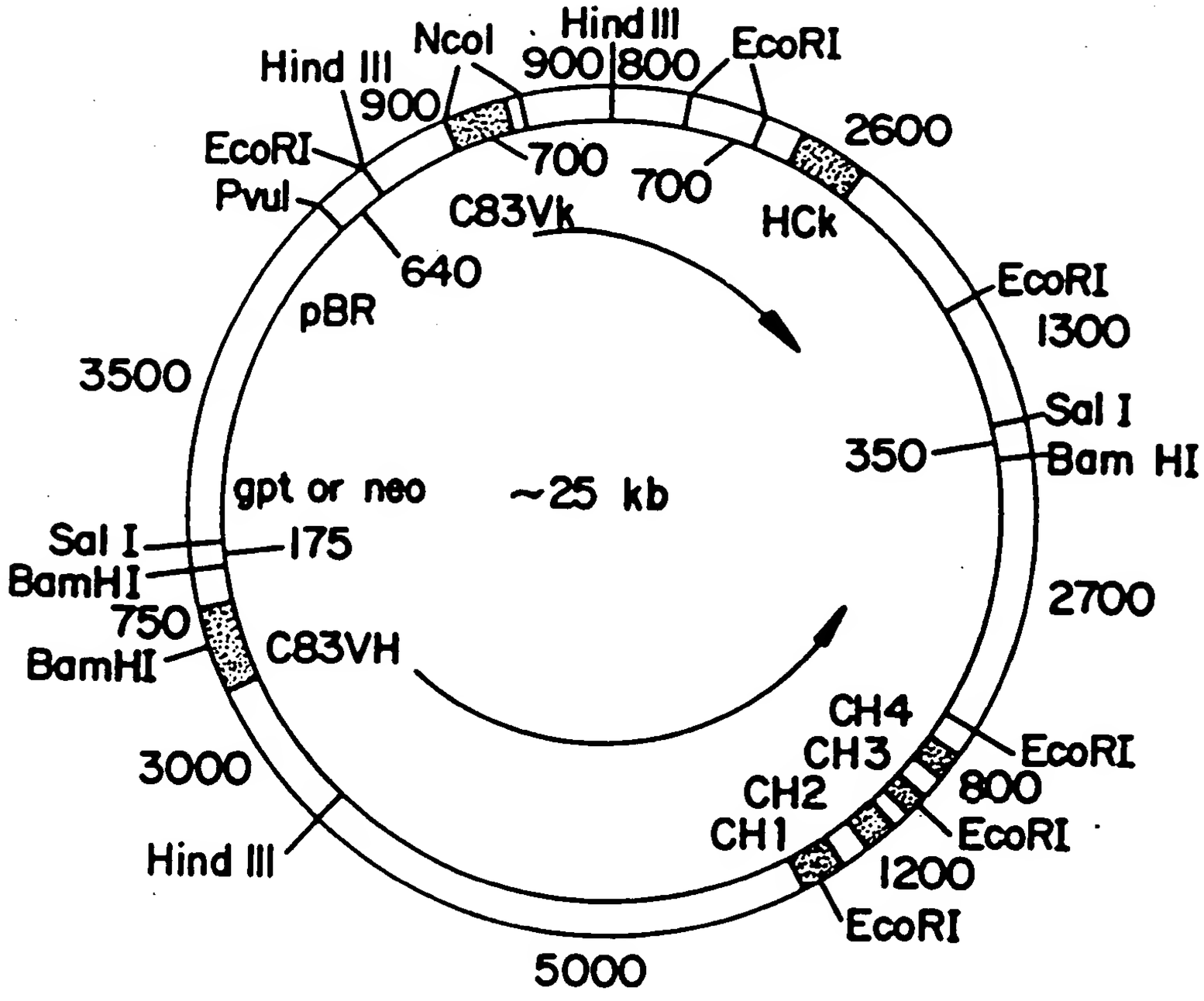
0
2
4
6
8
10
DAYS
CELL #C83
CELL #148

FIG. 4B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900
NcoI
Hind III
900
800
EcoRI
2600
700
700
EcoRI
Pvul
C83Vk
HCk
640
pBR
EcoRI
1300
3500
Sal I
350
Bam HI
gpt or neo
~25 kb
Sal I
175
BamHI
750
BamHI
C83VH
2700
CH4
CH3
EcoRI
3000
CH2
800
CH1
EcoRI
1200
Hind III
EcoRI
5000

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05322

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL) 16 August 1989, see page 4, line 35 - line 44; page 8, line 37 - line 38	1,2,5-8, 11-13, 15-19, 24
X	Science, Vol. 229, September 1985, S. L. Morrison: "Transfectomas Provide Novel Chimeric Antibodies ", see page 1202 - page 1207 see page 1206, second column, second paragraph and page 1204	1-3,5-8, 11-13, 15-19, 24
Y	EP, A1, 0125023 (GENENTECH, INC. ET AL.) 14 November 1984, see page 18, line 15; page 27, line 13	1-3,5-13,15-21,24, 26

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	11. 01. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Alfredo Prein

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A3, 0173494 (THE BOARD OF TRUSTEES OF THELELAND STANFORD JUNIORUNIVERSITY) 5 March 1986, see page 10, line 15 - line 20; page 11, line 33; page 12, line 12; page 20	1-3,5-13,15-21,24, 25
A	Nature, Vol. 312, December 1984, G. L. Boulianne et al.: "Production of functional chimaeric mouse/human antibody ", see page 643 - page 646	1-3,5-13,15-21,24, 25
A	Nature, Vol. 312, December 1984, M. S. Neuberger et al.: "Recombinant antibodies possessing novel effector functions ", see page 604 - page 608 see page 605, right column and fig 1	1-3,5-13,15-21,24, 25
A	EP, A2, 0315062 (BRISTOL-MYERS COMPANY) 10 May 1989, see page 6-8 and claims	1-3,5-13,15-21
A	Science, Vol. 229, August 1985, Jean L. Marx: "Antibodies Made to Order ", see page 455 - page 456 see page 455, bridging paragraph between second and third column	1-3,5-13,15-21
A	EP, A1, 0314161 (BRISTOL-MYERS COMPANY) 3 May 1989, see page 3, line 20; page 16, line 23 see claims	1-3,5-13,15-21
P,X	EP, A2, 0388964 (BOEHRINGER MANNHEIM GMBH) 26 September 1990, see page 3, lines 16-29 and 32	1,11

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	National Library of Medicine, File Med 88, Accession no. 88297774, Ramachandra RN: "Human--Human hybridomas secreting lipid A reactive monoclonal antibodies", Immunol Lett 1988 Jun;18(2):93-7	31
Y		26
	--	
X	EP, A1, 0271379 (ROUSSEL-UCLAF) 15 June 1988, see claims	31
Y		26
	--	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/05322

SA 40359

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0328404	16/08/89	AU-D-	3062689	06/09/89
		GB-A-	2216126	04/10/89
		WO-A-	89/07452	24/08/89
EP-A1- 0125023	14/11/84	AU-B-	598441	28/06/90
		AU-D-	2642984	11/10/84
		AU-D-	5201390	30/08/90
		CA-A-	1218613	03/03/87
		JP-A-	60155132	15/08/85
		US-A-	4816567	28/03/89
EP-A3- 0173494	05/03/86	AU-D-	4655685	06/03/86
		JP-A-	61119196	06/06/86
EP-A2- 0315062	10/05/89	AU-D-	2444688	27/04/89
		JP-A-	2000477	05/01/90
EP-A1- 0314161	03/05/89	AU-D-	2436388	01/06/89
		JP-A-	2138989	28/05/90
EP-A2- 0388964	26/09/90	DE-A-	3909708	27/09/90
EP-A1- 0271379	15/06/88	FR-A-B-	2606421	13/05/88
		JP-A-	1137993	30/05/89